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Low immunogenicity of the common lipoamide dehydrogenase subunit (E3) of mammalian pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase multienzyme complexes

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The production of high-titre monospecific polyclonal antibodies against the purified pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase multienzyme complexes from ox heart is described. The specificity of these antisera and their precise reactivities with the individual components of the complexes were examined by immunoblotting techniques. All the subunits of the pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase complexes were strongly antigenic, with the exception of the common lipoamide dehydrogenase component (E3). The titre of antibodies raised against E3 was, in both cases, less than 2% of that of the other subunits. Specific immunoprecipitation of the dissociated *N*-[^{3}H]ethylmaleimide-labelled enzymes also revealed that E3 alone was absent from the final immune complexes. Strong cross-reactivity with the enzyme present in rat liver (BRL) and ox kidney (NBL-1) cell lines was observed when the antibody against ox heart pyruvate dehydrogenase was utilized to challenge crude subcellular extracts. The immunoblotting patterns again lacked the lipoamide dehydrogenase band, also revealing differences in the apparent M_r of the lipoate acetyltransferase subunit (E2) from ox kidney and rat liver. The additional 50000-M_r polypeptide, previously found to be associated with the pyruvate dehydrogenase complex, was apparently not a proteolytic fragment of E2 or E3, since it could be detected as a normal component in boiled sodium dodecyl sulphate extracts of whole cells. The low immunogenicity of the lipoamide dehydrogenase polypeptide may be attributed to a high degree of conservation of its primary sequence and hence tertiary structure during evolution.

The multienzyme complexes that catalyse the lipoic acid-mediated oxidative decarboxylation of pyruvate and 2-oxoglutarate have been isolated from microbial and eukaryotic cells as functional units of high M_r [for reviews see Koike & Koike (1976) and Reed & Oliver (1981)].

The overall oxidative decarboxylation reaction can be represented as:

$$R\text{–}CO\text{–}CO_2H + CoA\text{-}SH + NAD^+ \rightarrow R\text{–}CO\text{–}S\text{-}CoA + CO_2 + NADH + H^+$$

where $R = CH_3$ (pyruvic acid) or $HO_2C\text{–}[CH_2]_2$ (2-oxoglutaric acid).

The available evidence suggests that in eukaryotic cells the complete process occurs within the mitochondrial matrix–inner-membrane compartment via a co-ordinated sequence of reactions. These reactions are: decarboxylation of the 2-oxo acid, reductive acylation of the lipoyl moiety and reoxidation of the dihydrolipoyl moiety with NAD^+ as the ultimate electron acceptor.

Abbreviation used: SDS, sodium dodecyl sulphate.

The ox heart pyruvate dehydrogenase complex consists of three catalytic components: pyruvate decarboxylase (E1) (EC 1.2.4.1), lipoate acetyltransferase (E2) (EC 2.3.1.12) and lipoamide dehydrogenase (E3) (EC 1.6.4.3) (Linn *et al.*, 1972). Enzyme E1 contains two types of non-identical subunits of M_r 42000 (α) and 37000 (β), which form a tetramer ($\alpha_2\beta_2$). The structural core of the complex is formed by the lipoate acetyltransferase (E2), to which several copies of E1 and E3 are non-covalently bound. E2 consists of a single type of polypeptide chain whose M_r has been estimated by means of SDS/polyacrylamide-gel electrophoresis to be between 70000 and 74000 (Barrera *et al.*, 1972; Hamada *et al.*, 1975; Machicao & Wieland,

1980). This estimation is at variance with the value of 52000 reported by Barrera *et al.* (1972) on the basis of electron microscopy, symmetry considerations, sedimentation-equilibrium analysis and gel filtration in 6M-guanidinium chloride (Kresze *et al.*, 1980). Enzyme E3 is composed of two identical subunits. Each one contains a molecule of FAD and has an M_r value of 55000 [for reviews see Reed & Pettit (1981) and Wieland (1983)]. An additional component of M_r 50000–52000 has been observed by SDS/polyacrylamide-gel electrophoresis of the purified pyruvate dehydrogenase complex in the Laemmli system (Stanley & Perham, 1980; Kresze & Steber, 1979; Kresze *et al.*, 1980).

The subunit composition of the 2-oxoglutarate dehydrogenase complex from ox heart has also been established (Koike & Koike, 1976). The complex is composed of a lipoate succinyltransferase core (E2) (EC 2.3.1.6) to which the 2-oxoglutarate decarboxylase (E1) (EC 1.2.4.2) and the lipoamide dehydrogenase (E3) components are bound. Enzyme E1 is a homodimer with subunits of M_r 96000, whereas the E2 component is composed of a single type of polypeptide, of M_r 48000 (Linn, 1971).

The lipoamide dehydrogenase is also a component of the branched-chain 2-oxo acid dehydrogenase complex involved in the metabolism of isoleucine, leucine and valine (Lawson *et al.*, 1983).

The mammalian pyruvate dehydrogenase complex is regulated by a phosphorylation–dephosphorylation mechanism. In the presence of MgATP, the E1 component is phosphorylated by a kinase that is intrinsic to the complex and tightly bound to the core enzyme. The phosphorylation causes loss of the activity of the enzyme. Dephosphorylation and re-activation are accomplished by a mitochondrial phosphatase, loosely associated with the complex (Reed, 1981; Randle, 1981). A comparable regulatory system has been described for the branched-chain 2-oxo acid dehydrogenase complex (Cook *et al.*, 1983). However, there is no evidence that the 2-oxoglutarate dehydrogenase complex is modulated by covalent modification.

Owing to the key positions that the reactions catalysed by these complexes occupy in metabolism, the structural and regulatory aspects of these complexes have been studied in several laboratories (Denton *et al.*, 1975). However, fewer efforts have been directed towards an understanding of the mechanisms of biosynthesis, transport and assembly of these macromolecular aggregates inside the mitochondria (Chii-Whei *et al.*, 1983).

The production of well-characterized specific antibodies against the whole complexes and their individual subunits is a necessary step to undertake these studies. The present paper deals with the preparation of specific antibodies against the 2-oxoglutarate dehydrogenase and pyruvate dehydrogenase complexes and describes some of the immunological characteristics of these multienzyme aggregates.

Materials and methods

Materials

Mops, leupeptin (propionyl-L-leucyl-L-leucyl-L-arginal), substrates, coenzymes, protein A, Triton X-100 and Tween 20 (polyethylene sorbital monolaureate) were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.) Poly(ethylene glycol) 6000 was purchased from Serva (Heidelberg, West Germany). Nitrocellulose paper (0.45 μm pore size) was from Schleicher und Schüll (Dassel, West Germany). Iodogen was obtained from Pierce Laboratories (Rockford, IL, U.S.A.).

Eagle's medium (Glasgow modification) was purchased from Flow Laboratories, Irvine, Scotland, U.K. The marker proteins for M_r determination ('low-molecular-weight' standards) came from Bio-Rad Laboratories (Richmond, CA, U.S.A.).

N-[*Et*-2-^{3}H]Ethylmaleimide (50Ci/mmol) was purchased from New England Nuclear (Boston, MA, U.S.A.).

Enzymes

Pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase complexes were purified from ox heart basically as described by Stanley & Perham (1980), with the following modifications. All operations were carried out at 4°C, and always started with fresh ox hearts. After the first poly(ethylene glycol) precipitation step, all buffered solutions, before gel filtration, were supplemented with 0.15 μM-leupeptin and 0.5% (v/v) rat serum. After the purification on Sepharose CL-2B, the enzymes were pelleted by centrifugation at 176000*g* in a Beckman Ti60 rotor for 2h for the pyruvate dehydrogenase complex and for 5h for the 2-oxoglutarate dehydrogenase complex. The complexes were dissolved in 50mM-Mops buffer, pH7.0, containing 2.7mM-EDTA and 0.1mM-dithiothreitol and stored at −20°C in small portions after mixing with 0.33 vol. of glycerol.

In this way, we obtained both enzymes with specific activities of 120–200nkat/mg when assayed at 25°C. The extent of cross-contamination was estimated to be less than 1% on the basis of activity.

Assays

The overall activity of the 2-oxo acid dehydrogenase complexes was determined by monitoring NADH formation at 340nm and 25°C as described by Brown & Perham (1976).

Inactivation of pyruvate dehydrogenase complex by ATP was carried out as described by Hucho *et al.* (1972)

Protein was determined by a modification of the Lowry procedure as described by Markwell *et al.* (1976), with bovine serum albumin as a standard.

Preparation of antibodies

Highly purified fractions of the 2-oxo acid dehydrogenase complexes were used as antigens. Purity was estimated to be between 95 and 98% by densitometric scanning of the Coomassie Blue-stained polyacrylamide gels of the isolated complexes after electrophoresis. Pyruvate dehydrogenase complex (1mg) dissolved in 50mM-Mops buffer, pH7.0, containing 2mM-EDTA and 0.1mM-dithiothreitol was mixed with an equal volume of Freund's complete adjuvant and injected subcutaneously at multiple sites in the neck and back of a 4-month-old New Zealand White rabbit. Similar amounts of enzyme were administered at 2–3-week intervals thereafter. At 10 days after the fourth injection, blood was obtained from a marginal ear vein. Additional booster injections were administered at approximately 1-month intervals with 0.5mg of protein mixed with incomplete Freund's adjuvant. The serum was collected, divided into small samples and kept frozen at −20°C until use. A similar regime was followed for the production of the antiserum against the 2-oxoglutarate dehydrogenase complex.

SDS/polyacrylamide-gel electrophoresis and fluorography

Electrophoretic analysis of proteins was carried out on 10% (w/v) polyacrylamide-gel slabs (19cm × 9.5cm) with 5% (w/v) stacking gel with the discontinuous buffer system of Laemmli (1970). Gels were stained with 0.04% (w/v) Coomassie Brilliant Blue R for 8–12h and destained in 10% (v/v) acetic acid. Samples of proteins were boiled for 5min in 62.5mM-Tris/HCl buffer, pH6.8, containing 2% (w/v) SDS, 10% (w/v) sucrose, 5% (v/v) 2-mercaptoethanol and 0.001% Pyronin Y (Laemmli sample buffer) before being applied to the gels.

Apparent M_r values were calculated with reference to the mobility of the following proteins, which were included in each electrophoretic run: phosphorylase *a* (M_r 92000), bovine serum albumin (M_r 67000), ovalbumin (M_r 45000), carbonic anhydrase (M_r 31000), soya-bean trypsin inhibitor (M_r 21000) and lysozyme (M_r 14000). Fluorography was performed as described by Bonner & Laskey (1974).

Isolation of subcellular fractions from cultured cells

Buffalo-rat liver cells (BRL) were grown in Glasgow-modified Eagle's medium supplemented with 10% (v/v) calf serum, 100 munits of penicillin/l and 37 munits of streptomycin/l.

Cell monolayers were washed twice with phosphate-buffered saline (0.15M-NaCl/20mM-sodium phosphate buffer, pH7.4) and then harvested in 10ml of ice-cold phosphate-buffered saline by scraping with a rubber 'policeman'. Samples were immediately dissociated by boiling in Laemmli sample buffer for 5min, and the remaining cells were fractionated as described by Attardi & Ching (1979) to obtain crude nuclear and mitochondrial pellets. Samples of these fractions were boiled as above. Any residual material was removed by centrifugation and the supernatant fractions were stored at −20°C.

Bovine kidney cells (NBL-1) were grown in the same medium utilized for the BRL cells supplemented with 10% (v/v) foetal-calf serum. Total cell extracts of the NBL-1 cells were prepared as described for the BRL cells.

Labelling of protein A

Protein A (1mg) dissolved in 1ml of 20mM-Tris/HCl buffer, pH7.2, containing 150mM-NaCl was labelled for 15min at room temperature with Iodogen (1mg) and 300–500μCi of $Na^{125}I$ (Salacinski *et al.*, 1981). [^{125}I]Iodide was removed by gel filtration on Sephadex G-25. Fractions containing the labelled protein A were pooled, divided into small samples and kept at −20°C until use.

Analysis of proteins by the immunoblotting technique

Subcellular fractions and purified proteins were subjected to SDS/polyacrylamide-gel electrophoresis. The resolved proteins were transferred electrophoretically on to nitrocellulose paper essentially as described by Towbin *et al.* (1979) for 2–4h at 400mA. The transfer buffer was supplemented with 0.02% (w/v) SDS. The nitrocellulose was then incubated with antiserum against the pyruvate dehydrogenase complex (diluted 1:100) or against the 2-oxoglutarate dehydrogenase complex (diluted 1:50), with the use of the modification suggested by Batteiger *et al.* (1982). Specific antigens were detected after incubation with ^{125}I-labelled protein A (60000–80000d.p.m./ml) to detect bound antibody. The dried blots were left at −80°C with X-Omat S film (Kodak), with intensifying screens to enhance the sensitivity of the procedure. Films were generally exposed for 2–3 days.

Labelling of 2-oxo acid dehydrogenase complexes with N-[^3H]ethylmaleimide

Thiol groups of dissociated pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase complexes were labelled with *N*-[^{3}H]ethylmaleimide as follows. A 100 μg portion of each protein was precipitated in 80% (v/v) acetone by storage at −20°C for several hours. The pellets were dissolved in 200 μl of 20 mM-Tris/HCl buffer, pH 7.2, containing 2% (w/v) SDS and then treated with 50 μCi of *N*-[^{3}H]ethylmaleimide. Samples were incubated for 30 min at room temperature before termination of the reaction by the addition of 2-mercaptoethanol to a final concentration of 5% (v/v). Labelled proteins were acetone-precipitated as before. The pellets were washed once with cold acetone, dried, redissolved in 200 μl of 2% (w/v) SDS and diluted 5-fold with Triton buffer [1% (v/v) Triton X-100/300 mM-NaCl/5 mM-EDTA/10 mM-Tris/HCl buffer, pH 7.4]. Finally, samples were stored at −20°C until required.

Immunoprecipitation

Immunoprecipitation of the ^{3}H-labelled proteins was carried out in a final volume of 100 μl, containing 1 μg (approx. 150000 d.p.m.) of the labelled complexes in Triton buffer. The pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase complexes were immunoprecipitated with 5 μl and 10 μl of their respective antisera. In both cases, appropriate controls were included by replacing the antisera with serum obtained from a non-immunized rabbit.

After the addition of antibody, the samples were incubated for 1 h at room temperature and then for 12–16 h at 4°C. At this stage, 100 μl of 10% (w/v) fixed *Staphylococcus aureus* cells, Cowan I strain (Calbiochem-Behring Corp., Los Angeles, CA, U.S.A.), were added and the incubation was continued for 2 h at room temperature. The pellets were washed three times with Triton buffer and once with the same buffer without detergent. Finally, pellets were dissociated by boiling with 40–50 μl of Laemmli (1970) sample buffer and the extracts were loaded on 10% (w/v) polyacrylamide gel slabs. Under the conditions described, 70–80% of the original radioactivity was recovered in the immunoprecipitates.

Results

The final specific activities of the purified pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase complexes ranged from 120 to 200 nkat/mg (six preparations). These values compare favourably with the values reported by Stanley & Perham (1980) for the enzymes prepared by the same procedure. However, our highest recoveries of 50 μkat/kg of heart for the pyruvate dehydrogenase complex and 12 μkat/kg of heart for the 2-oxoglutarate dehydrogenase complex are lower than the recoveries obtained by the same authors. We also observed an increased instability of the 2-oxoglutarate dehydrogenase complex at later stages in the purification. The inclusion of rat serum and leupeptin at early stages during the purification improved the yield of this complex.

The susceptibility of the E2 component to contaminating proteolytic enzymes, leading to dissociation and inactivation of the complex, has been established previously (Linn, 1971; Kresze *et al.*, 1981). The addition of rat serum and leupeptin to the buffer media provides further protection of the enzymes against endogenous proteinases (Lynen *et al.*, 1978).

Subunit composition and immunoblotting pattern of the 2-oxoglutarate dehydrogenase complex

Fig. 1 (lanes 2–5) shows the subunit composition of the 2-oxoglutarate dehydrogenase complex obtained when decreasing amounts of protein were examined by SDS/polyacrylamide-gel electrophoresis. The profile shows three major components with M_r values of 96000 (E1), 55000 (E3) and 48000 (E2). Similar values have been reported for the ox heart enzyme by Linn (1971). Small amounts of contaminating material (5–10%), most of which probably results from degradation by endogenous proteinases, were also present.

As shown in Fig. 1 (lanes 6–9), the antiserum raised against the whole complex is capable of detecting very small amounts of the 2-oxoglutarate dehydrogenase components E1 and E2, but no reaction with the E3 subunit is observed even with larger amounts of the protein (lane 6).

Subunit composition and immunoblotting pattern of the pyruvate dehydrogenase complex

Fig. 2 (lanes 5–8) shows an analogous profile of the components of the pyruvate dehydrogenase complex detected after polyacrylamide-gel electrophoresis. The M_r values of the main bands are estimated to be 70000 (E2), 55000 (E3), 42000 (E1α) and 36000 (E1β). An additional band of M_r 50000, migrating below the E3 component, has been consistently found in our purified preparations of the complex when the electrophoretic analysis is performed with the Laemmli buffer system. The presence of this protein in preparations of the pyruvate dehydrogenase complex has been noted previously by Stanley & Perham (1980) and Kresze *et al.* (1980).

This component (which we call 'component X') has not yet been identified. Results not given in the present paper suggest that the protein is tightly

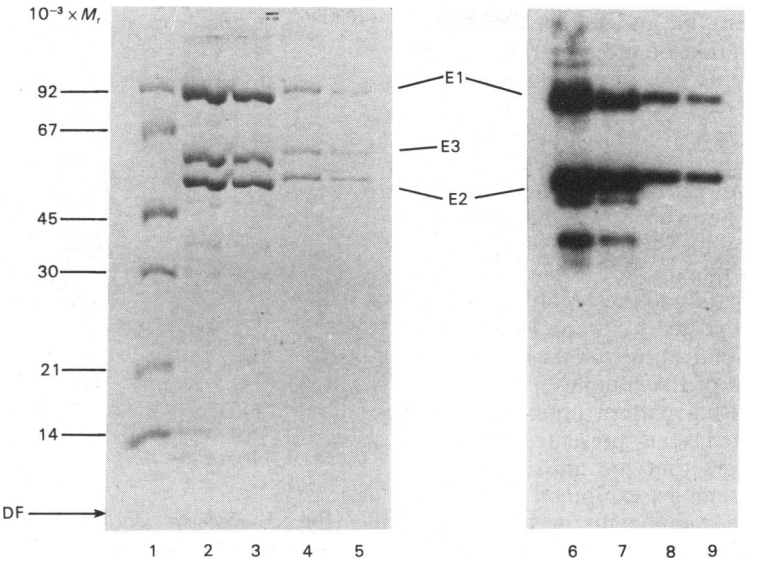

Fig. 1. *Reactivity of antiserum to ox heart 2-oxoglutarate dehydrogenase with the individual subunits of the complex*
Various amounts of purified 2-oxoglutarate dehydrogenase were subjected to SDS/polyacrylamide-slab-gel electrophoresis. One half of the duplicate 10% (w/v) gel was stained with Coomassie Blue (lanes 1–5); polypeptides on the other half were transferred electrophoretically on to nitrocellulose paper and processed for immunodetection of the antigenic polypeptides (lanes 6–9) by autoradiography. Lane 1, M_r markers; lanes 2 and 6, 10 μg of protein; lanes 3 and 7, 5 μg; lanes 4 and 8, 1 μg; lanes 5 and 9, 0.5 μg. DF is the dye front. See the Materials and methods section for further details.

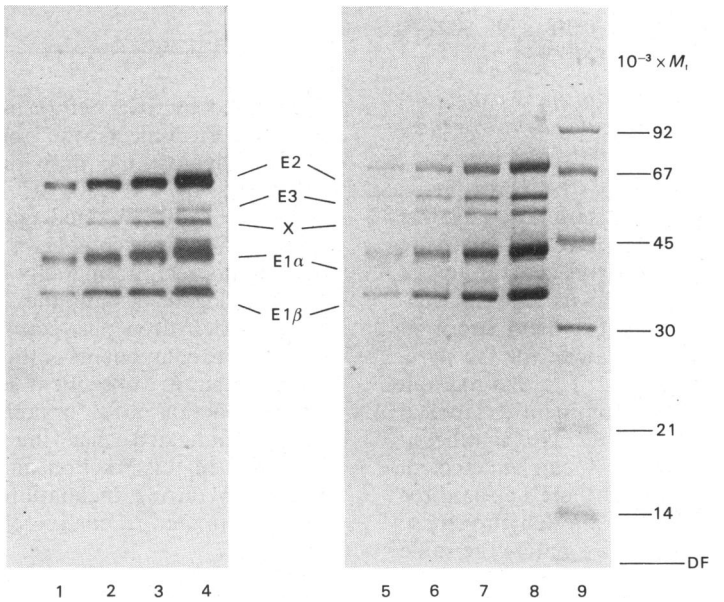

Fig. 2. *Reactivity of antiserum to ox heart pyruvate dehydrogenase with the constituent polypeptides of the complex*
Various amounts of purified pyruvate dehydrogenase multienzyme complex were subjected to electrophoresis on SDS/10%-(w/v)-polyacrylamide slab gels. Half of the gel was used for transferring the resolved subunits on to nitrocellulose paper (lanes 1–4); the duplicate half was stained with Coomassie Blue (lanes 5–8). After incubation with specific antiserum, the immune complexes were 'decorated' with ^{125}I-labelled protein A before autoradiography (see details in the Materials and methods section). Lanes 1–4, 0.3 μg, 0.75 μg, 1.5 μg and 3 μg of protein respectively. Lanes 5–8 contained 3.3 times more enzyme (1 μg, 2.5 μg, 5 μg and 10 μg) than their counterparts in lanes 1–4 to facilitate direct visible staining with Coomassie Blue. Lane 9, M_r markers.

bound to the core enzyme and does not result from proteolytic degradation of the E2 or E3 components, since there is no immunological cross-reactivity with these polypeptides.

The presence of pyruvate dehydrogenase kinase in our preparation of pyruvate dehydrogenase complex was demonstrated by total inactivation of the complex after 15 min at 30°C in the presence of 0.2 mM-ATP and by measuring the incorporation of $^{32}P_i$ from [^{32}P]ATP into the complex. However, the bands corresponding to the M_r of the subunits of the kinase, as reported by Stepp *et al.* (1983), were not observed, indicating that the kinase is not a major component of the complex.

The immunoblotting pattern obtained for the pyruvate dehydrogenase is presented in Fig. 2 (lanes 1–4). It shows that the antiserum raised against the whole complex exhibits a high reactivity against all the components of the complex with the exception of the lipoamide dehydrogenase. When low amounts of the complex were loaded on the gel (lane 1), the band corresponding to the lipoamide dehydrogenase was not detected, whereas the bands of the other components of the complex were clearly seen. We have estimated that the minimal amount of whole complex required to detect E3 (0.75–1.5 μg) is about 50 times higher than the amount required to detect the other constituent subunits, E1α, E1β and E2, which are visible at amounts of 0.01–0.03 μg (not shown) under the same conditions of exposure.

N-[^{3}*H*]*Ethylmaleimide labelling of the 2-oxoglutarate dehydrogenase and pyruvate dehydrogenase complexes*

To facilitate the analysis of the components immunoprecipitated by the antisera raised against the 2-oxoglutarate dehydrogenase and pyruvate dehydrogenase complexes, the free thiol groups of these enzymes were labelled with *N*-[^{3}H]ethylmaleimide. Fig. 3 demonstrates the pattern of radiolabelled polypeptides obtained for the pyruvate dehydrogenase complex. The fluorograph indicates that the most prominently labelled component is the E1α subunit. No significant incorporation into 'component X' can be detected. In contrast, as shown in Fig. 4 (lane 1), the three components of the 2-oxoglutarate dehydrogenase complex were labelled to approximately the same extent.

Immunoprecipitation of the 2-oxoglutarate dehydrogenase and pyruvate dehydrogenase complexes

The subunit polypeptides of the 2-oxoglutarate dehydrogenase and pyruvate dehydrogenase complexes that were specifically immunoprecipitated by the antiserum directed against each complex are shown in Fig. 4. Since the complexes were

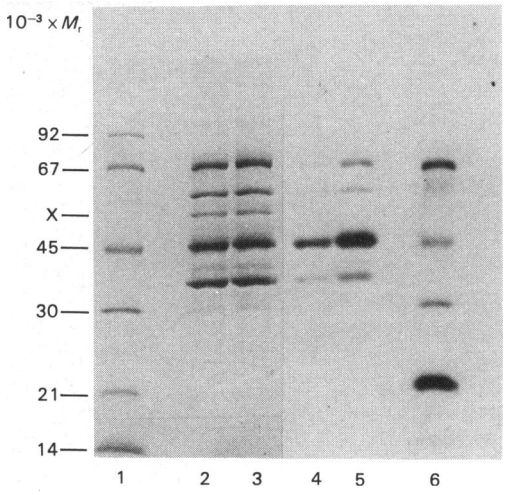

Fig. 3. *Subunit labelling of pyruvate dehydrogenase multienzyme complex with N-*[^{3}*H*]*ethylmaleimide*
Samples of SDS-dissociated pyruvate dehydrogenase complex (15 μg of protein) were mixed with ^{3}H-labelled enzyme, 5000 d.p.m. (lanes 2 and 4) or 10000 d.p.m. (lanes 3 and 5), before resolution on a 10% (w/v) polyacrylamide slab gel. After staining with Coomassie Blue (lanes 1–3), the gel was processed for fluorography (lanes 4–6). ^{125}I-labelled M_r markers (10 μg) were included in lanes 1 and 6. The minor band with M_r 38000 may be a proteolytic fragment of E2.

dissociated by SDS before immunoprecipitation, this pattern reflects the relative titres of the antisera against the individual subunits of the complexes.

The antiserum directed against the 2-oxoglutarate dehydrogenase complex is capable of effectively immunoprecipitating only subunits E1 and E2. In an analogous fashion, the antiserum against the pyruvate dehydrogenase complex immunoprecipitated all the components of the complex apart from the E3 subunit. Subsequent analysis of the supernatant fraction after immunoprecipitation (not shown) indicated that the intact E3 subunit remained in this fraction and was not degraded selectively during incubation with antiserum or formalin-treated *S. aureus* cells.

Immunological detection of pyruvate dehydrogenase complex in cell extracts

Fig. 5 reveals that the anti-(pyruvate dehydrogenase) serum is capable of detecting the components of the complex present in several cell extracts with high specificity and sensitivity. The antigenic components were detectable in SDS extracts of total rat liver (BRL) or bovine kidney (NBL-1) cells and exclusively in the mitochondrial fraction

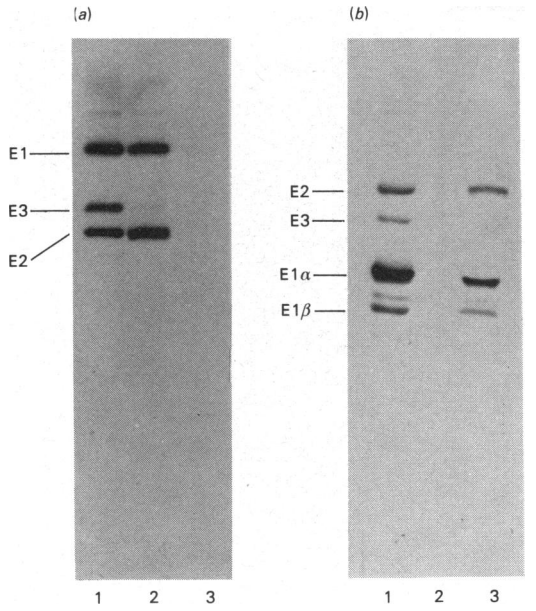

Fig. 4. *Immunoprecipitation of subunits from dissociated 2-oxoglutarate dehydrogenase and pyruvate dehydrogenase multienzyme complexes*

2-Oxoglutarate dehydrogenase and pyruvate dehydrogenase complexes labelled with *N*-[^{3}H]ethylmaleimide were incubated with their respective antisera. Immune complexes were then adsorbed on formalin-treated *S. aureus* cells before fluorographic analysis after separation by electrophoresis on SDS/10% (w/v) polyacrylamide gels (see the Materials and methods section). (*a*) Lane 1, ^{3}H-labelled 2-oxoglutarate dehydrogenase; lane 2, immunoprecipitate of lane 1 with anti-(2-oxoglutarate dehydrogenase) serum; lane 3, control immunoprecipitate with pre-immune serum. (*b*) Lane 1, ^{3}H-labelled pyruvate dehydrogenase; lane 2, immunoprecipitate after incubation with control serum; lane 3, immunoprecipitate with anti-(pyruvate dehydrogenase) serum.

of BRL cells. The most obvious feature of the immunoblotting patterns obtained with the various subcellular fractions is the complete absence of the lipoamide dehydrogenase component.

The M_r values of the bovine and rat lipoate acetyltransferase (E2) subunits are also clearly different, in agreement with the values for the mammalian E2 subunit, estimated to be in the range 70000–76000 for the bovine kidney and heart enzymes and 68000 for the rat heart enzyme (Matuda *et al.*, 1983).

An important additional finding (Fig. 5) is that the 50000-M_r species of unknown function is located in the mitochondrial compartment.

Discussion

A major observation in the present work is that the common lipoamide dehydrogenase component (E3) of the 2-oxoglutarate dehydrogenase and pyruvate dehydrogenase multienzyme complexes elicits an extremely poor immunological response relative to the other constituent polypeptides. Evidence supporting this conclusion has been obtained by (*a*) analysis of the precise reactivity of the antisera by immunoblotting versus the purified ox heart enzymes or crude extracts of bovine kidney or rat liver cell lines and (*b*) by direct immunoprecipitation of the dissociated multienzyme complexes, in which E3 is seen to be excluded specifically from the final immune complex. In contrast, immunoprecipitates of the native 2-oxo acid dehydrogenase complexes (not shown) contain the full complement of polypeptides, since E3 is still associated with the 'core' enzyme E2 under normal conditions. Matuda *et al.* (1983) have also reported the preparation of antibodies against the rat heart pyruvate dehydrogenase complex. However, this group have not characterized their antisera by employing the refined and sensitive techniques of the present study.

What is the basis for the low immunogenicity of the E3 subunit polypeptide? As this component is present on the surface(s) of both multimolecular aggregates (Bleile *et al.*, 1981) and can be released without disrupting the organization of the 'core' enzyme E2, it is clearly accessible to the immune system. Moreover, it is not possible to attribute this phenomenon to a low content of E3 in the purified complexes. In pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase, the polypeptide chain proportions of E1, E2 and E3 polypeptides are 5:5:1 and 1:2:1 respectively. In pyruvate dehydrogenase the 50000-M_r component appears, from densitometric analysis of a Coomassie Blue-stained polyacrylamide gel after electrophoresis, to be present in similar amounts to E3, but has an antibody titre at least 20-fold higher than the lipoamide dehydrogenase (Figs. 2 and 5).

In our opinion the most plausible interpretation of these data is that the low immunogenicity of E3 reflects the high degree of conservation of amino acid sequence in this polypeptide. The complete sequence of the lipoamide dehydrogenase component in *Escherichia coli* has been established by analysis of the cloned gene (Stephens *et al.*, 1983). Interestingly, considerable homologies are detected in comparing the sequence of the *E. coli* enzyme with those of tryptic peptides of the enzyme from pig heart. Since E3 is a common component of three multienzyme aggregates, it appears feasible that it has been highly conserved during evolution

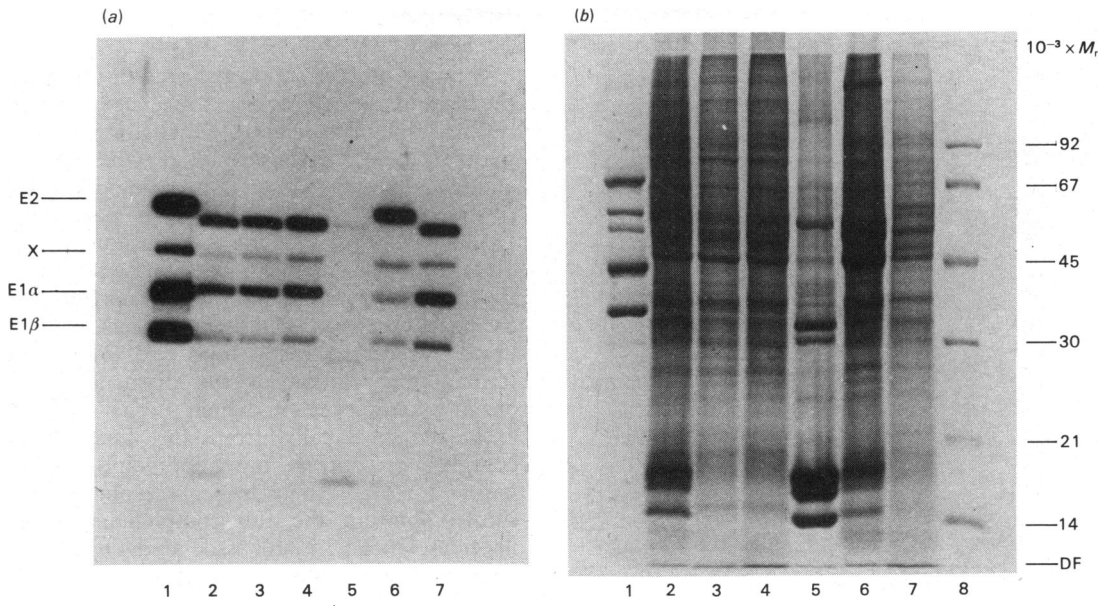

Fig. 5. *Immunodetection of pyruvate dehydrogenase polypeptides in whole-cell extracts and cellular subfractions*
Samples of purified pyruvate dehydrogenase complex and various cell extracts were electrophoresed in duplicate on SDS/10% (w/v) polyacrylamide gels. Half of the slab gel was employed for transference of the polypeptides on to nitrocellulose paper and subsequent immunoblotting analysis (*a*); the other half was stained with Coomassie Blue (*b*). (*a*) and (*b*) Lane 1, pyruvate dehydrogenase, 0.4 μg and 10 μg respectively; lane 2, SDS extract of BRL cells (80 μg); lanes 3 and 4, post-nuclear supernatant fraction (20 μg and 40 μg respectively); lane 5, BRL-cell nuclei (50 μg); lane 6, SDS extract of NBL-1 cells (100 μg); lane 7, BRL-cell mitochondria (40 μg); lane 8, M_r markers. DF is the dye front.

owing to the severe constraints imposed on amino acid substitution by the multiple (and differing) specific subunit interactions involved in these structurally and functionally analogous 2-oxo acid dehydrogenase complexes.

Two further points of interest emerge from these studies with respect to the pyruvate dehydrogenase complex. Firstly, there is a clear difference in the mobility of the E2 subunit on SDS/polyacrylamide-gel electrophoresis in comparing the bovine heart, bovine kidney (NBL-1 cells) and rat liver (BRL cells) enzymes. It is apparent that the E2 subunit in rat liver has an M_r approx. 5000 lower than that of the equivalent bovine subunit polypeptide. This difference is observed consistently between the rat and bovine lipoyl transacetylases from liver, heart or kidney, indicating that it is a species-related rather than a tissue-related phenomenon. Consistent with this finding, the M_r values for the rat heart and bovine heart E2 polypeptides have been reported as 68000 and 70000–76000 respectively. However, the immunoblotting procedure provides a sensitive probe for detecting subtle qualitative and quantitative differences in specific enzymes from a variety of tissues or organisms.

Secondly, immunoanalysis of the profile of pyruvate dehydrogenase subunits in boiled SDS extracts of whole cells, i.e. after resolution by electrophoresis on SDS/polyacrylamide gels, has permitted us to conclude that the tightly associated undesignated 50000-M_r species is a normal cellular component, since proteolytic degradation of the complex was avoided (unless occurring naturally *in vivo*). In addition, its location in the mitochondrial compartment (Fig. 5) also argues against the possibility that it is a protein that becomes adventitiously bound to the pyruvate dehydrogenase complex during its isolation. Further characterization of this polypeptide and its possible function in relation to the multienzyme complex is required.

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References

Attardi, G. & Ching, E. (1979) *Methods Enzymol.* **56G**, 66–79

Barrera, C. R., Nahimira, G., Hamilton, L., Munk, P., Elley, M. H., Linn, T. C. & Reed, L. J. (1972) *Arch. Biochem. Biophys.* **148**, 343–358

Batteiger, B., Newhall, W. J. & Jones, R. B. (1982) *J. Immunol. Methods* **55**, 297–307

Bleile, D. M., Hackert, M. L., Pettit, F. H. & Reed, L. J. (1981) *J. Biol. Chem.* **256**, 514–519

Bonner, W. M. & Laskey, R. A. (1974) *Eur. J. Biochem.* **46**, 83–88

Brown, C. R. & Perham, R. N. (1976) *Biochem. J.* **155**, 419–427

Chii-Whei, C. H., Utter, M. F. & Patel, M. S. (1983) *J. Biol. Chem.* **258**, 2315–2320

Cook, K. G., Lawson, R. & Yeaman, S. J. (1983) *FEBS Lett.* **157**, 59–62

Denton, R. M., Randle, P. J., Bridges, B. J., Cooper, R. H., Kerbey, A. L., Pask, H. T., Severson, D. L., Stansbie, D. & Whitehouse, S. (1975) *Mol. Cell. Biochem.* **9**, 27–53

Hamada, H., Otsuka, K., Tanaka, N., Ogasahara, K., Koike, K., Hiraoka, T. & Koike, M. (1975) *J. Biochem. (Tokyo)* **78**, 187–197

Hucho, F., Randall, D. D., Roche, T. E., Burgett, M. W., Pelley, J. W. & Reed, L. J. (1972) *Arch. Biochem. Biophys.* **151**, 328–340

Koike, M. & Koike, K. (1976) *Adv. Biophys.* **9**, 187–227

Kresze, G.-B. & Steber, L. (1979) *Eur. J. Biochem.* **95**, 569–578

Kresze, G.-B., Dietl, B. & Ronft, H. (1980) *FEBS Lett.* **112**, 48–50

Kresze, G.-B., Ronft, H., Dietl, B. & Steber, L. (1981) *FEBS Lett.* **127**, 157–160

Laemmli, U.K. (1970) *Nature (London)* **227**, 680–685

Lawson, R., Cook, K. G. & Yeaman, S. J. (1983) *FEBS Lett.* **157**, 54–58

Linn, T. C. (1971) *Arch. Biochem. Biophys.* **161**, 505–514

Linn, T. C., Pelley, J. W., Pettit, F. H., Hucho, F., Randall, D. D. & Reed, L. J. (1972) *Arch. Biochem. Biophys.* **148**, 327–342

Lynen, A., Sedlaczek, E. & Wieland, O. H. (1978) *Biochem. J.* **169**, 321–328

Machicao, F. & Wieland, O. H. (1980) *Hoppe-Seyler's Z. Physiol. Chem.* **361**, 1093–1106

Markwell, M. A., Haas, S., Bieber, L. L. & Tolbert, N. E. (1976) *Anal. Biochem.* **87**, 206–210

Matuda, S., Shirahama, T., Saheki, T., Miura, S. & Mori, M. (1983) *Biochim. Biophys. Acta* **741**, 86–93

Randle, P. J. (1981) *Curr. Top. Cell. Regul.* **18**, 107–129

Reed, L. J. (1981) *Curr. Top. Cell Regul.* **18**, 95–105

Reed, L. J. & Oliver, R. M. (1981) *Adv. Exp. Med. Biol.* **148**, 231–241

Reed, L. J. & Pettit, F. H. (1981) *Cold Spring Harbor Conf. Cell Proliferation* **8**, 701–711

Salacinski, P. R. P., McLean, C., Sykes, J. E. C., Clement-Jones, V. V. & Lowry, P. J. (1981) *Anal. Biochem.* **117**, 136–146

Stanley, C. & Perham, R. N. (1980) *Biochem. J.* **191**, 147–154

Stephens, P. E., Lewis, H. M., Darlison, M. G. & Guest, J. R. (1983) *Eur. J. Biochem.* **135**, 219–239

Stepp, L. R., Pettit, F. H., Yeaman, S. J. & Reed, L. J. (1983) *J. Biol. Chem.* **258**, 9454–9458

Towbin, H., Staehelin, T. & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4350–4354

Wieland, O. H. (1983) *Rev. Physiol. Biochem. Pharmacol.* **96**, 124–170